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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 1041WO	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/EP 03/14576	International filing date *(day/month/year)* 18.12.2003	Priority date *(day/month/year)* 20.12.2002
International Patent Classification (IPC) or both national classification and IPC C12N15/82		
Applicant GREENOVATION BIOTECH GMBH et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 7 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the opinion
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
14.07.2004	26.04.2005
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized Officer Huber, A Telephone No. +49 89 2399-8173

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP 03/14576

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:

Description, Pages

1-47 as originally filed

Sequence listings part of the description, Pages

1-13 as originally filed

Claims, Numbers

1-42 received on 12.03.2005 with letter of 10.03.2005

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).

   ☐ the language of publication of the international application (under Rule 48.3(b)).

   ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   ☒ contained in the international application in written form.

   ☒ filed together with the international application in computer readable form.

   ☐ furnished subsequently to this Authority in written form.

   ☐ furnished subsequently to this Authority in computer readable form.

   ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:

   ☐ the claims, Nos.:

   ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)).

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-42
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-42
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-42
	No:	Claims	

2. Citations and explanations

see separate sheet

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. The following documents are referred to:

   D1: WO 01/29242 A (MONSANTO CO) 26 April 2001 (2001-04-26)
   D2: KOPRIVOVA ANNA ET AL: "Functional knockout of the adenosine 5'-phosphosulfate reductase gene in Physcomitrella patens revives an old route of sulfate assimilation." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 277, no. 35, 30 August 2002 (2002-08-30), pages 32195-32201, XP002245177 August 30, 2002 ISSN: 0021-9258
   D3: SCHAEFER D G ET AL: "EFFICIENT GENE TARGETING IN THE MOSS PHYSCOMITRELLA PATENS" PLANT JOURNAL, BLACKWELL SCIENTIFIC PUBLICATIONS, OXFORD, GB, vol. 11, no. 6, June 1997 (1997-06), pages 1195-1206, XP000885764 ISSN: 0960-7412
   D4: WO 01/25456 A (GORR GILBERT ;RESKI RALF (DE); GREENOVATION PFLANZENBIOTECHNO (DE)) 12 April 2001 (2001-04-12)
   D5: LEROUGE P ET AL: "N-glycoprotein biosynthesis in plants: recent developments and future trends" PLANT MOLECULAR BIOLOGY, NIJHOFF PUBLISHERS, DORDRECHT, NL, vol. 38, 1998, pages 31-48, XP002140796 ISSN: 0167-4412

2. **Novelty (Art. 33(2) PCT):**

   None of the cited documents discloses a bryophyte cell or a method to produce such a cell that comprises a dysfunctional fucosyl transferase nucleotide sequence and a dysfunctional xylosyl transferase nucleotide sequence.
   Therefore the subject-matter of Claims 1-42 is considered novel.

3. **Inventive step (Art. 33(3) PCT):**

   Document D1 which is considered to be the closest prior art, discloses plant cells, in

particular maize cells and tobacco cells, that have been modified to express a human or bovine beta-galactosidase transferase in order to produce heterologous proteins with mammalian glycans. On pages 8-9, it is also suggested to reduce one or more of the plant's own glycosyl transferases, in particular xylosyl transferase and fucosyl transferase activity, by antisense strategy (example 4, page 77).
Present application differs from the teaching of D1 in that the xylosyl transferase activity and the fucosyl transferase activity of the bryophyte have been completely abolished, while in D1 only one of the plant gycosyltransferase activities has been reduced by antisense strategy (see p. 78, l. 4-7).
In view of D1 the problem underlying the present application is the provision of a plant, resp. a bryophyte which has completely lost the capability to transfer plant specific fucose and xylose residues on the core structure of N-glycans without the loss of the complex N-glycan structures in order to ensure a humanized N-glycosylation structure on glycoproteins expressed in plants/bryophytes.
The problem has been solved by knocking out the fucosyl and xylosyl transferase of the bryophyte and to transform the cell with mammalian beta-1,4 galT.

No prior art documents discloses or suggests the double knock out of the two genes. By applying the antisense method as disclosed in D1, no complete loss of enzyme function can be expected.
A pointed out by the applicant, at the priority date of present application it was expected that a complete knock out of enzyme function would result in a lethal phenotype since the enzymes are essential for regeneration, differentiation and/or development. Also it was considered that isoenzymes were able to compensate each other as disclosed by Bakker et al, FEBS Letters 507; 307-3312, 2001) and that N-glycan processing could be by-passed by other members of the glycosyltransferase family.

Therefore, in view of the above considerations the skilled person would not have been motivated to completely knock-out the resp. genes but rather would have followed the approach of D1.

The IPEA is therefore of the opinion that the claims directed to bryophyte cells which comprise a dysfunctional fucosyl transferase and a dysfunctional xylosyl transferase involve the required inventive step (see however the comments on clarity, point 4.1,

below).

Claims 14-29 relate to a method for producing a bryophyte cell wherein fucT and xylT activity is substantially reduced. Although in its present drafting the method encompasses the antisense approach of D1, an inventive step has been acknowledged since in D1 the activity of only one of the two genes is reduced (in addition see the comments, point 4.3 below).
For the same reason also Claims 30-42 can be considered inventive.

4. **Comments on the clarity of the claims**

4.1. The term "dysfunctional" is not clear (Art. 6 PCT) since it does not unambiguously exclude partially functional genes. For assessing inventive step the term has been interpreted in the light of the description on p. 6, l. 6-10 and p. 7, l. 16-22).

4.2 Claims 2-4 are directed to a transformed bryophyte cell which, according to claim 1, should be incapable of modifying plant N-linked glycans, but which drives expression of a glycosylated protein. This is only possible if the bryophyte cell is additionally transformed with functional galactosyl transferase. The claims therefore lack an essential technical feature which is necessary to put the invention into practice. The same objection applies to Claims 15-17.

4.3 Claims 14-29 are directed to a method of producing a bryophyte cell wherein fucT and xylT activity is substantially reduced.
According to the description the essential technical feature of present application is the complete knock-out of fucT and xylT activity in order to ensure that the heterologous proteins produced in bryophytes do not contain of the fucosyl or xylosyl residues. Partially inactivated enzymes would not solve the problem underlying the application, namely the provision of proteins with "humanized" glycosylation pattern. In addition, it should be noted that the term "substantially" is a relative term with no well recognized meaning (Art. 6 PCT).

4.4 Claim 30 does not meet the requirements of Article 6 PCT in that the matter for which protection is sought is not clearly defined. The claim attempts to define the subject-matter in terms of the result to be achieved, which merely amounts to a statement of

the underlying problem, without providing the technical features necessary for achieving this result.

CLAIMS

1. A transformed bryophyte cell that comprises i) a dysfunctional fucosyl transferase nucleotide sequence and ii) a dysfunctional xylosyl transferase nucleotide sequence.

2. A transformed bryophyte cell according to claim 1, wherein the said cell further comprises a nucleotide sequence operably linked to an exogenous promoter that drives expression in the said bryophyte cell, wherein said nucleotide sequence encodes a glycosylated polypeptide that is expressed in the bryophyte cell.

3. A transformed bryophyte cell according to claim 2, wherein said glycosylated polypeptide comprises animal glycosylation patterns.

4. A transformed bryophyte cell according to claim 3, wherein said glycosylated polypeptide comprises mammalian glycosylation patterns.

5. A transformed bryophyte cell according to any one of claims 1 to 4, further comprising a nucleotide sequence operably linked to an exogenous promoter that drives expression in the said bryophyte cell, wherein said nucleotide sequence encodes a functional mammalian galactosyl transferase that is expressed in the bryophyte cell.

6. A transformed bryophyte cell according to claim 5, wherein the mammalian galactosyl transferase that is expressed is a beta-1,4 galT.

7. A transformed bryophyte cell according to claim 6, wherein the mammalian galactosyl transferase that is expressed is a human beta-1,4 galT.

8. A bryophyte cell according to any one of claims 1 to 7, wherein the bryophyte cell is selected from species of the genera *Physcomitrella*, *Funaria*, *Sphagnum*, *Ceratodon*, *Marchantia* and *Sphaerocarpos*.

9. A bryophyte cell according to claim 8, wherein the bryophyte cell is selected from *Physcomitrella*.

10. A bryophyte cell according to claim 9, wherein the bryophyte cell is from *Physcomitrella patens*.

11. A bryophyte cell according to any one of claims 2 to 10, wherein the mammalian glycosylated polypeptide is selected from the group comprising a polypeptide having a primary amino acid sequence of a human glycosylated polypeptide, a primary amino acid sequence of a non-human mammalian glycosylated protein, and/or a primary amino acid sequence of an antibody or an active fragment thereof.

12. A bryophyte cell according to claim 11, wherein the mammalian glycosylated polypeptide is a human polypeptide.

13. A bryophyte cell according to claim 11 or 12, wherein the mammalian glycosylated polypeptide is selected from the group consisting of human insulin, preproinsulin, VEGF, proinsulin, glucagon, interferons such as alpha-interferon, beta-interferon, gamma-interferon, blood-clotting factors selected from Factor VII, VIII, IX, X, XI, and XII, fertility hormones including luteinising hormone, follicle stimulating hormone growth factors including epidermal growth factor, platelet-derived growth factor, granulocyte colony stimulating, prolactin, oxytocin, thyroid stimulating hormone, adrenocorticotropic hormone, calcitonin, parathyroid hormone, somatostatin, erythropoietin (EPO), and enzymes such as beta-glucocerebrosidase, haemoglobin, serum albumin, and collagen.

14. A method of producing at least a bryophyte cell wherein fucT and xylT activity is substantially reduced, that comprises introducing into the said cell i) a first nucleic acid sequence that is specifically targeted to an endogenous fucosyl transferase nucleotide sequence and ii) introducing into the said cell a second nucleic acid sequence that is specifically targeted to an endogenous xylosyl transferase nucleotide sequence.

15. A method according to claim 14, wherein the said transformed bryophyte cell further comprises a nucleotide sequence operably linked to an exogenous promoter that drives expression in the said bryophyte cell, wherein said nucleotide sequence encodes a glycosylated polypeptide that is expressed in the bryophyte cell.

16. A method according to claim 15, wherein said glycosylated polypeptide comprises animal glycosylation patterns.

17. A method according to claim 16, wherein said glycosylated polypeptide comprises mammalian glycosylation patterns.

18. A method according to any one of claims 14 to 17, further comprising introducing into the said cell an isolated nucleic acid sequence that comprises nucleic acid operably linked to an exogenous promoter that drives expression in a bryophyte cell, wherein said nucleic acid encodes a functional mammalian galactosyl transferase polypeptide.

19. A method according to claim 18, wherein the galactosyl transferase nucleotide sequence is a beta-1,4 galactosyl transferase (beta-1,4 galT) nucleotide sequence.

20. A method according to claim 19, wherein the galactosyl transferase nucleotide sequence is a human beta-1,4 galactosyl transferase (beta-1,4 galT) nucleotide sequence.

21. A method according to any one of claims 15 to 20, wherein the mammalian glycosylated polypeptide is selected from the group comprising a protein having a primary amino acid sequence of a human protein, a primary amino acid sequence of a non-human mammalian protein, and/or a primary amino acid sequence of an antibody or an active fragment thereof.

22. A method according to any one of claims 15 to 21, wherein the glycosylated polypeptide is selected from the group consisting of human insulin, preproinsulin, proinsulin, glucagon, interferons such as alpha-interferon, beta-interferon, gamma-interferon, blood-clotting factors selected from Factor VII, VIII, IX, X, XI, and XII, fertility hormones including luteinising hormone, follicle stimulating hormone growth factors including epidermal growth factor, platelet-derived growth factor, granulocyte colony stimulating, prolactin, oxytocin, thyroid stimulating hormone, adrenocorticotropic hormone, calcitonin, parathyroid hormone, somatostatin, erythropoietin (EPO), and enzymes such as beta-glucocerebrosidase, haemoglobin, serum albumin, collagen, and human and non-human proteins selected from amidases, amylases, carbohydrases, cellulase, dextranase, esterases, glucanases, glucoamylase, lactase, lipases, pepsin, peptidases, phytases, proteases, pectinases, casein, whey proteins, soya proteins, gluten and egg albumin.

23. A method according to any one of claims 14 to 22, wherein the bryophyte cell is selected from species of the genera *Physcomitrella*, *Funaria*, *Sphagnum*, *Ceratodon*, *Marchantia* and *Sphaerocarpos*.

24. A method according to claim 23, wherein the bryophyte cell is selected from *Physcomitrella*.

25. A method according to claim 24, wherein the bryophyte cell is from *Physcomitrella patens*.

26. A method according to any one of claims 15 to 25, wherein the mammalian glycosylated polypeptide is selected from the group comprising a polypeptide having a primary amino acid sequence of a human glycosylated polypeptide, a primary amino acid sequence of a non-human mammalian glycosylated protein, and/or a primary amino acid sequence of an antibody or an active fragment thereof.

27. A method according to claim 26, wherein the mammalian glycosylated polypeptide is a human polypeptide.

28. A method according to claim 6 or 27, wherein the mammalian glycosylated polypeptide is selected from the group consisting of human insulin, preproinsulin, VEGF, proinsulin, glucagon, interferons such as alpha-interferon, beta-interferon, gamma-interferon, blood-clotting factors selected from Factor VII, VIII, IX, X, XI, and XII, fertility hormones including luteinising hormone, follicle stimulating hormone growth factors including epidermal growth factor, platelet-derived growth factor, granulocyte colony stimulating, prolactin, oxytocin, thyroid stimulating hormone, adrenocorticotropic hormone, calcitonin, parathyroid hormone, somatostatin, erythropoietin (EPO), and enzymes such as beta-glucocerebrosidase, haemoglobin, serum albumin, and collagen.

29. A method according to any one of claims 15 to 28, wherein the exogenous promoter is selected from inducible, chemical-regulated, constitutive or cell specific promoters.

30. A nucleic acid vector suitable for producing at least a bryophyte cell wherein fucosyl and xylosyl transferase nucleotide sequences are dysfunctional.

31. A nucleic acid vector according to claim 30 that comprises i) a first nucleic acid sequence that is specifically targeted to an endogenous fucosyl transferase nucleotide sequence and ii) a second nucleic acid sequence that is specifically targeted to an endogenous xylosyl transferase nucleotide sequence.

32. A nucleic acid vector according to claim 30 or 31, further including a polynucleotide that encodes a functional mammalian glycosyl transferase for use in a method according to any of claims 15 to 29.

33. A nucleic acid vector according to claim 32, wherein said polynucleotide encodes a recombinant mammalian galactosyl transferase.

34. A nucleic acid vector according to claim 33, wherein said polynucleotide encodes a recombinant human beta-1,4 galactosyl transferase.

35. A host cell containing a nucleic acid vector according to any one of claims 30 to 34.

36. A host cell according to claim 35 which is a bryophyte cell.

37. A host cell according to claim 35 which is a prokaryote cell.

38. A method of producing a host cell according to any of claims 35 to 37, the method including incorporating said nucleic acid vector into the cell by means of transformation.

39. Use of a nucleic acid vector according to any one of claims 30 to 34 in the production of a transgenic bryophyte cell.

40. A host cell according to claim 35 or 36 which is comprised in a bryophyte, or a bryophyte part, or an extract or derivative of a bryophyte or in a bryophyte cell culture.

41. A bryophyte plant or bryophyte tissue comprising a bryophyte cell according to any one of claims 1 to 13, 36 and 40.

42. A method of producing a bryophyte plant, the method including incorporating a nucleic acid vector according to any of claims 30 to 34 into a bryophyte cell and regenerating a bryophyte from said cell.